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Isolation, screening and characterization of crude glycerol utilizing microorganism for the production of 1, 3-propanediol

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A B S T R A C T

Rapidly Expanding market for biodiesel has increased the supply and eventually decreased the cost of crude glycerol, which makes an attractive sustainable feed stock for the fuel and chemical Industry. The conversion of glycerol may be carried out by both chemical as well as biological means. Many microorganisms are known to convert glycerol to value added industrially important products. The present study involved the isolation of potent bacterial strains from soil sample obtained from biodiesel information and Demonstration centre, Gulbarga University, Gulbarga, Karnataka, India. The isolated strain was *Enterobacter safensis* and is designated as GNTEW-13. As the *Enterobacter safensis* strain is the known producer of 1,3-PDO, is an important component of fuel and polyesters (used widely in the petroleum industry) using biodiesel derived crude glycerol for producing 1,3-Propanediol, which is a substituent in various industrial sectors as good and remarkable solution from an commercial as well as ecological point of view.

Introduction

1,3,Propanediol, an emerging bulk chemical, is a monomer of producing plastics with special properties such as Biodegradability and is the base of new Polyester (Biebl *et al.,* 1999). Demand of the biodiesel and soap industries have raised in the world market since 1998. The production of glycerol (1kg of glycerol is produced with every 10 kg of biodiesel) as s by-product of these industries is increasing, this trend has made glycerol less expensive.

For this reason glycerol is being considered a good feed stock for industrial fermentation. Glycerol can be converted to various components trough bioconversion, like 1,3-PDO which is used to produce polymer like polytrimethylene tetraphthalate (PTT) (Biebl *et al.,* 1999). Current commercial 1, 3-PDO production is based on chemical Production, this process requires harmful chemical solvents, high

temperature and high pressure condition, and expensive catalyst (Cheng *et al.,* 2005).

Much attention has been paid to biosynthesis because it use only renewable feed stock and does not generate harmful byproduct compared with chemical production (Cheng *et al.,* 2005; Gonzalez-Pajuel *et al.,* 2004). In converting glycerol to 1,3-PDO many microorganisms such as *Klebsiella, Enterobacteriaceae, Clostridium, Citrobacter,* and *Lactobacillus*. Due to their high yield and productivity, *Klebsiella pneumoniae, Clostridium butyricum* are currently considered the most promising microorganism for industrial process among the other microorganisms (Anand *et al.,* 2011).

Materials and Methods

Sample collection

Soil samples were collected from various region of Gulbarga district, Karnataka. And the crude glycerol was collected from Biodiesel Information and Demonstration Centre (BIDC), Gulbarga University, Gulbarga, Karnataka.

Enrichment of soil microorganisms

The medium used for bacterial enrichment contained following components (%, w/v): glycerol-2.0, yeast extract-0.5, peptone-1.0, NaCl-0.9 and pH 7.0. This medium was based on LB (Luria-Bertani) medium which was used for bacterial culture. About 1.0 g of soil sample was inoculated to a 250 ml flask with 50 ml enrichment medium and then incubated aerobically at 37^0C on a rotator shaker continuously at about 150 rpm. After the completion of one day incubation period, nearly about 100 µl of bacterial culture was transferred to another flask with the same enrichment medium and incubated overnight. Thus the bacteria that could use glycerol as carbon resource and grow quickly under aerobic condition would be enriched. After four rounds of such enrichment operation, 1.0 ml of 10^{-8} fold diluted culture broth was plated on agar plates and incubated at 37^0C overnight. The medium used for pour plate was the same as enrichment medium, but only 2.0% (w/v) of agar was added. Visual evaluation of colonies was used for the selection of bacterial strains for further identification and characterization.

Isolation of bacterial strains from soil sample

The crude glycerol utilizing microorganisms from soil sample were selected by plating on mineral salt medium which contain the following composition (%, w/v) $NaNO_3$-0.4, NaCl-0.1, KCl-0.1, $Cacl_2$ $2H_2O$-0.01, KH_2PO_4-0.3, $Na_2HPO_4.H_2O$-0.3, $MgSO_4$-0.02, $FeSO_4.7H_2O$-0.0001, trace element-0.2 ml, crude glycerol as a only carbon resource and pH 6.8. The composition of trace elements was (%, w/v): $FeCl_3.6H_2O$-0.008, $ZnSO_4.7H_2O$-0.075, $CoCl_2.6H_2O$-0.008, $CuSO_4.5H_2O$-0.0075, $MnSO_4.H_2O$-0.075, H_3BO_3-0.015 and $Na_2MoO_4.2H_2O$-0.005. Total of 17 isolates were selected that can utilize only crude glycerol as carbon source.

Screening of microbes for 1, 3-PDO production

Total of 12 isolates were screened for their ability of 1,3-PDO production in 250ml of Erlenmeyer flask containing 100ml of production medium at 35^0C, 150 rpm for 24 h under aerobic and anaerobic conditions. The production medium contained (%, w/v): glycerol-2.0, K_2HPO_4-.0069, KH2PO4-0.025, $(NH_4)_2SO_4$-0.4, $MgSO_4.7H_2O$-0.02, yeast extract-0.15 and 1.0ml of trace elements solution. The composition of trace

elements was (%, w/v): $MnSO_4.4H_2O$-0.01, $ZnCl_2$-0.007, $Na_2MoO_4.2H_2O$-0.003, H_3BO_3 -0.006, $CoCl_2.6H_2O$ -0.02, $CuSO_4.5H_2O$-0.002, $NiCl_2.5H_2O$-0.002, 0.2 ml $FeSO_4$ solution (Hao *et al.,* 2008). The composition ferrous sulfate solution was (%w/v): $FeSO_4.7H_2O$-0.5.

Based on Thin Layer Chromatography (TLC) strains were selected.

The isolates which were showing more 1,3-PDO production were designated as GNTEW-13-I, GNTEW-13-II, GNTEW-13-III, GNTEW-13-IV, GNTEW-13-V, GNTEW-13-VI, GNTEW-13-VII, GNTEW-13-VIII, GNTEW-13-IX, GNTEW-13-X, GNTEW-13-XI and GNTEW-13-XII.

Further studies were carried out to characterize the above strains.

Optimization of growth condition

The production media was used to optimize the cultural media of isolated bacteria. The pH was adjusted to 5.5, 7, 8, 9 and 11.0 in production medium.

For optimization of incubation period and temperature the culture plates were incubated at 25 and 37°C for 18–96 hours.

Characterization of 1,3-PDO producing isolates

The morphological, physical and biochemical characterizations of GNTEW-13-I, GNTEW-13-II, GNTEW-13-III, GNTEW-13-IV, GNTEW-13-V, GNTEW-13-VI, GNTEW-13-VII, GNTEW-13-VIII, GNTEW-13-IX, GNTEW-13-X, GNTEW-13-XI and GNTEW-13-XII were investigated.

Fermentation experiment for the production of 1,-propanediol

Culture Conditions

After obtaining approximately 12 isolates, fermentation experiments were performed to determine their ability to produce 1,3-propanediol from glycerol. The isolates were cultivated in Hungate test tubes or Erlenmeyer flasks in the appropriate-ate cultivation media (37°C, 24 h). All the isolation treatments of bacteria from the genus *Enterobacter Safensis* were performed in a chamber for the cultivation of anaerobic microorganisms. Isolates from the genus *Enterobacter Safensis* was cultured under relative anaerobic conditions in an incubation chamber. In the next stage, production media, enriched with pure or crude glycerol (80.0 ± 1.0 g/L) and a pH indicator (1% solution of bromocresol purple, 2.0 mL/L), were inoculated. The pH was regulated at 7.0 using 20% NaOH. The temperature of the fermentation process was 32°C.

Analytical Methods

Samples of fermentation broth were collected and centrifuged at 10,000 × g for 10 min, and the cell-free supernatants were filtered through syringe filters prior to HPLC analysis. A Hewlett Packard 1050 system equipped with a refractive index detector was used. Analyses were performed isocratically at a flow rate of 0.6 mL/min on an Aminex HPX-87H 300 × 7.8 column (Bio-Rad, CA, USA) at a constant temperature of 65°C. H_2SO_4 (0.5 ml) was the mobile phase. External standards were applied for identification and quantification.

The cell concentration (g/L) was determined using a linear equation derived from the relationship of cell dry weight (90°C until constant weight) and the optical density (OD) at 600 nm (Analytik Jena Specord 50). The cells were collected by centrifugation (10,000 × g for 10 min) in a Hereaus Biofuge centrifuge (Eppendorf) and washed twice with distilled water.

Phylogenetic Analyses

The sequences encoding the 16S rRNA of *Enterobacter safensis* strain GNTEW-13-1 & GNTEW-13-5 were compared with the randomly selected sequences *in* GenBank. The sequences were aligned using the Crustal-W program as implemented in BioEdit (version 7.0.9). The phylogenetic analyses were conducted using the MEGA 4.0 software [31]. The neighbour-joining method was used for phylogenetic reconstruction, and the p-distance was used for distance analysis. The best phylogenetic distance tree is shown.

Results and Discussion

A purple color band was seen on TLC plates, when TLC plates (loaded with sample and run in mobile phase) were allowed to react with vanillin reagent. As it was already studied that vanillin reagent specifically chemically react with "OH" group of 1,3-PDO and gives purple color band. According to the paper (Rajendra Kumar *et al.,* 2012) the RF value of Standard 1,3-PDO was found to be 0.62± 0.5.So on the basis of purple color band and RF values in the range of 0.62±0.5 was used to screened out 1,3-PDO producing isolates from the total 17 isolates.

The bacteria were stained as a deep blue in color when observed under microscope, and showing various shape and arrangement of the respective isolates.

When smear was observed under microscope, one type of colonies was found i.e. blue or violet. The bacteria which retain the primary stain (appear dark blue or violet) are called gram-positive and those that lose the crystal violet and counter stained by safranin (appear red) are referred as gram-negative.

GNTEW-13-I, GNTEW-13-III, GNTEW-13-V, GNTEW-13-VI, GNTEW-13-VII they were rod shaped and arranged in single random manner hence, they were designated as bacillus. *GNTEW-13-II and GNTEW-13-XII* was rod shaped and arranged in clumps hence, they were designated as *Staphylobacilli*.

GNTEW-13-IX was found to be rod shaped but arranged in chain pattern hence, designated as *Streptobacilli*. *GNTEW-13-IV and GNTEW-13-VIII* was found to be spherical in shape and arranged in clumps hence, designated as *Staphylococci. GNTEW-13-IX and GNTEW-13-X* was found to be spherical in shape and arranged in single random manner hence, designated as cocci.

The optimum condition for the better growth of these 4 isolates *(GNTEW-13-III, GNTEW-13-IV, GNTEW-13-V and GNTEW-13-VIII)* were found to be pH -7.0 to 8.0, temperature- 30^0C to 37^0C and salt concentration from 1.5% to 2.0%. Hence, optimization was done successfully.

The plates were examined for various Biochemical test to know the biochemical nature of the isolated bacterial strains. The tubes were examined for the production of gas and acid. The production of gas in the Durham tube and change in color of broth (from red to yellow) due to the production of acid are shown in table 3.

Phylogenetic analyses

The obtained sequences were BLAST analyzed using nucleotide blast (blastn) of NCBI database (http://blast.ncbi.nlm. nih.gov) to know sequence similarity for obtained sequence.

The species identification was done by constructing phylogenetic tree using CLUSTALW2 (http://www.ebi.ac.uk/ Tools /msa/clustalw2) online tool as well as CLUSTAL X2 offline tool.

The present work on 1, 3-propandiol production by crude glycerol utilizing microorganism reveals that the collection of soil sample was done from various region of Gulbarga district, Karnataka. The biodiesel derived crude glycerol was collected from Biodiesel Information and Demonstration Centre (BIDC) unit, Gulbarga University, Gulbarga Karnataka. Total 17 crude glycerol utilizing isolates were screened out by culturing on Mineral salt Medium (Containing crude glycerol as carbon source). Out of 17 isolates, total 12 isolates were screened out for the production of 1, 3-PDO and they were designated as *GNTEW-13-I, GNTEW-13-II, GNTEW-13-III, GNTEW-13-IV, GNTEW-13-V, GNTEW-13-VI, GNTEW-13-VII, GNTEW-13-VIII, GNTEW-13-IX, GNTEW-13-X, GNTEW-13-XI and GNTEW-13-XII.* The total 12 isolates, which were showing positive results for 1,3-PDO production, were subjected to various characterization such as morphological characterization, physiological characterization and biochemical characterization. Out of 12 isolates *GNTEW-13-III, GNTEW-13-IV, GNTEW-13-V and GNTEW-13-VIII* were found to be more potential isolates as compared to the other.

In addition to this the optimization was done for *GNTEW-13-III, GNTEW-13-IV, GNTEW-13-V and GNTEW-13-VIII* to evaluate their optimum condition for the growth. Mainly two enzymes are responsible for conversion of crude glycerol to 1,3-PDO one is Glycerol dehydrates and another is 3-hydroxypropionaldehyde reductase.

Table.1 Bacterial characterization of isolated microbes

Isolates	Gram's staining results	Simple Staining results	
		Shape of bacteria	Arrangement of the bacteria
GNTEW-13-I	+	*Bacilli* (Rod shape)	Single
GNTEW-13-II	+	*Bacilli* (Rod shape)	Staphylo
GNTEW-13-III	+	*Bacilli* (Rod shape)	Single
GNTEW-13-IV	+	*Cocci* (spherical)	Staphylo
GNTEW-13-V	+	*Bacilli* (Rod shape)	Single
GNTEW-13-VI	+	*Bacilli* (Rod shape)	Single
GNTEW-13-VII	+	*Bacilli* (Rod shape)	Single
GNTEW-13-VIII	+	*Cocci* (spherical)	Staphylo
GNTEW-13-IX	+	*Cocci* (spherical)	Single
GNTEW-13-X	+	*Cocci* (spherical)	Single
GNTEW-13-XI	+	*Bacilli* (Rod shape)	Strepto
GNTEW-13-XII	+	*Bacilli* (Rod shape)	Staphylo

Table.2 Physiological response of isolates towards different physical parameters

Characteristics	GNTEW-13-III	GNTEW-13-IV	GNTEW-13-V	GNTEW-13-VI
I. Growth at different temperature				
Growth at 25^{0}C	-	-	+	+
Growth at 37^{0}C	+	+	+	+
II. Growth at different Ph				
Growth at pH 5.5	-	-	+	+
Growth at pH 7.0	+	+	+	+
Growth at pH 8.0	+	+	+	+
Growth at pH 9.0	+	-	+	-
Growth at pH 11.0	-	-	-	-
III. Growth on different NaCl concentration				
Growth on NaCl 1.5%	+	+	+	+
Growth on NaCl 2%	+	+	+	+
Growth on NaCl 5%	-	-	+	-
Growth on NaCl 7%	-	-	-	-
Growth on NaCl 10%	-	-	-	-

Table.3 Fermentation of sugar

Isolates	Glucose		Mannitol		sucrose	
	Acid	Gas	Acid	Gas	Acid	Gas
GNTEW-13-I	+	-	-	-	+	-
GNTEW-13-II	+	-	-	-	-	-
GNTEW-13-III	+	-	-	-	+	-
GNTEW-13-IV	+	-	-	-	-	-
GNTEW-13-V	+	+	+	+	+	+
GNTEW-13-VI	+	+	+	+	+	+
GNTEW-13-VII	+	+	+	+	+	+
GNTEW-13-VIII	+	-	-	-	-	-
GNTEW-13-IX	+	-	-	-	-	-
GNTEW-13-X	+	+	+	+	+	+
GNTEW-13-XI	+	-	+	+	-	-
GNTEW-13-XII	+	+	+	+	+	+

Figure.1 Screening of microbes for 1, 3-PDO production by Thin Layer Chromatography

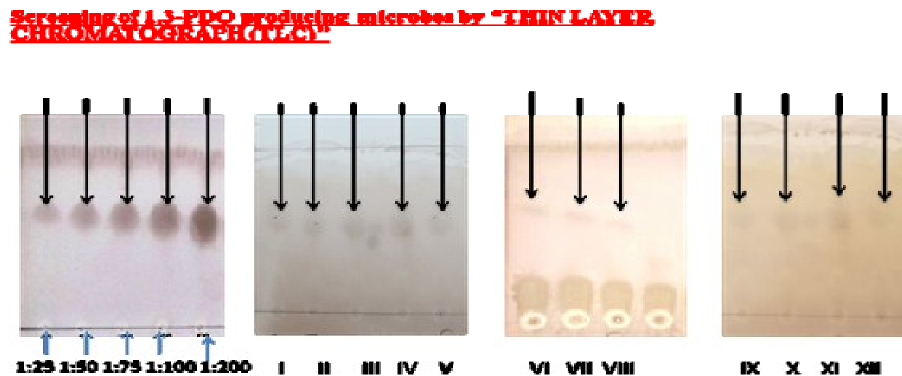
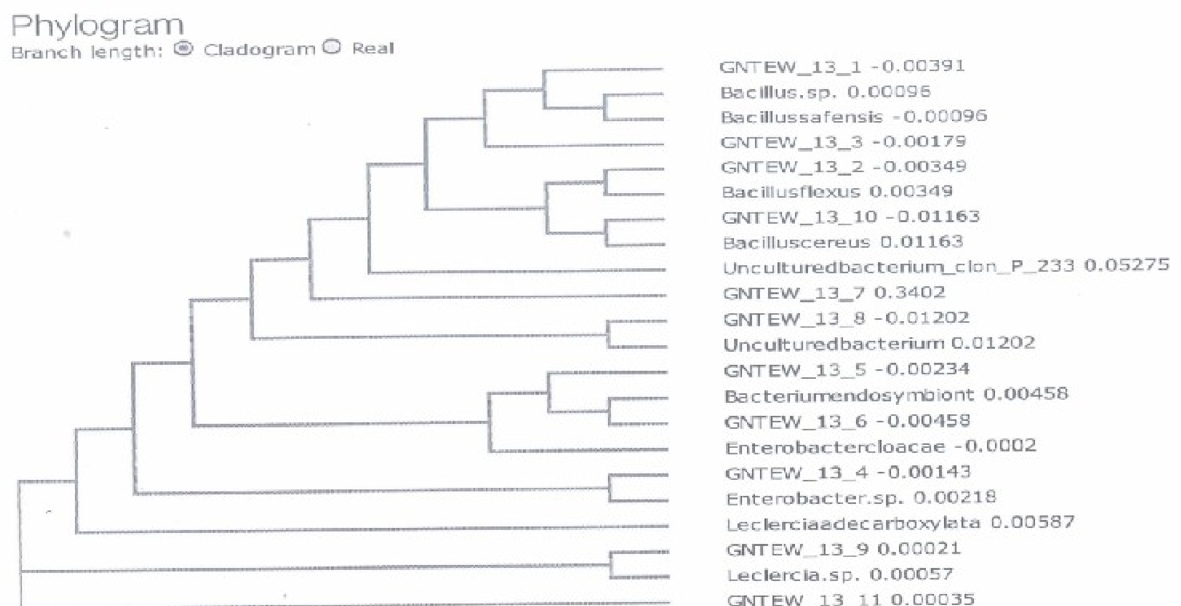


Figure.2 16S rRNA-based phylogenetic tree showing the position of glycerol-fermenting *Enterobacter safensis* among related *Enterobacters*



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